PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 1376	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/JP 03/15956	International filing date *(day/month/year)* 12.12.2003	Priority date *(day/month/year)* 12.12.2002
International Patent Classification (IPC) or both national classification and IPC C12Q1/68		
Applicant RIKEN et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09.07.2004	25.02.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Schwachtgen, J-L Telephone No. +49 89 2399-8933

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/JP 03/15956

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-33, 35, 36, 40, 42, 44-47	as originally filed
34, 37-39, 41, 43	filed with telefax on 04.10.2004

Sequence listings part of the description, Pages

1-2	as originally filed

Claims, Numbers

1-78	as originally filed

Drawings, Sheets

1/12-12/12	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 76-78

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (specify):

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 76-78

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the Standard.

☐ the computer readable form has not been furnished or does not comply with the Standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5-7, 21-24, 47
	No:	Claims	1-4, 8-20, 25-46, 48-75
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-75
Industrial applicability (IA)	Yes:	Claims	1-75
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: WO 02/31190 A (GENSET SA ;THILL GILBERT (FR)) 18 April 2002 (2002-04-18)

D2: KWON Y M ET AL: "Efficient amplification of multiple transposon-flanking sequences" JOURNAL OF MICROBIOLOGICAL METHODS, vol. 41, no. 3, August 2000 (2000-08), pages 195-199, XP002278628 ISSN: 0167-7012

1. The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1-4, 8-20, 25-46, 48-75 is not new in the sense of Article 33(2) PCT.

   The document D1 discloses a method of isolating and analysing nucleic acid mismatch regions of e.g. mutant isoforms comprising 1) denaturing and re-hybridising isoforms 2) removing single-stranded regions other than internal single-stranded regions and 3) selecting heteroduplexes comprising at least one internal single-stranded region with a single stranded trap (claims 1-6). The 3' or 5' overhangs are removed during step 2) with exonuclease VII (page). A reduction (fragmentation) step is performed at the selection step 3) by cutting double stranded nucleic acids with restriction endonucleases (page 23, line 33 - page 34, line 13).

   The disclosure in D1, thus anticipates all the technical features of the subject-matter of claims 1-4, 8-20, 25-46, 48-75.

3. Dependent claims 5-7, 21-24 and 47 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of Article 33(3) PCT in respect of inventive step, see document D2, where restriction enzymes having a 4 bp recognition site and Y-shaped linkers have been used in a similar method (Figure 1).

4. In his reply to the IPEA's Written Opinion, the Applicant has pointed out several alleged differences between the invention of the present application and the disclosure in D1. However, these differences are not reflected in the claims and are, thus, not taken into account for the establishment of the present report.

detail with reference to the following examples.

Example

Example 1

Protocol of Alternative Splicing Exon Library Method

Full-length cDNA libraries from cell line cultures of melanocyte and melanoma were constructed using the method developed by Carninci et al. Genome Res. 2000 Oct;10 (10); Carninci et al. Genomics. 2001 Sep;77 (1-2):79-90. We can use other method by developed by Maruyama, K., Sugano, S., 1994. Gene 138, 171-174. Lambda vector pFLCII (Derivative of the ampicillin-resistant plasmid pBlueScriptII-SK(+), Carninci et al., 2001, Genome Research, Vol.77, (1-2), 79-90). cDNA sequences were inserted into vector with the *Xho*I site at the 5' end of the cDNA and the *Bam*HI site at the 3' end.

We sequenced the 5' ESTs using the T7 primer and the 3' ESTs using the T3 primer. The following can be used for the library construction. Stock of the library-phage solution was made by adding 70 ml of DMSO (Dimethyl Sulfoxide, Wako Chemical, Japan) to 930 ml of phage solution and mixed gently manually. The stock was kept at - 80 degree C.

Part 1. DNA extraction from amplified phage.

1ml of phage stock solution was mixed gently with addition of RNase, 10u/μl and DNase, 1u/μl (both Promega), 2 μl of each enzyme, respectively. Solution was incubated on 37 degree C for 20 min. After that, 500μl of pre-swollen microgranular anion exchanger DE52 (Diethylamoioethyl cellulose, Whatman) was applied with keeping manual mixing for about 10min. Mixture was centrifuged for 1min at room

synthesized. After that, DNaseI (RQ1, RNase-free, Promega, 1u/μl) treatment was performed for about 30min: With addition of 20μl of 10mM $CaCl_2$ and 1μl of DNase. Sample was dissolved with 100μl of water and further purification with QIAGEN purification Kit(QIAGEN) was employed in accordance with the manufacturer's instructions. Final volume of solution was adjusted in 100μl of water. Then, proteinase K digestion was conducted followed by extraction with phenol/chloroform and chloroform, and cDNA was precipitated.

1st strand cDNA preparation.

A solution of 5μg of RNA sense strand (31μl) were combined to 5 μl of first-strand primer (SEQ ID NO:2) for a total volume of 36μl. (solution A). 5μg of RNA antisense strand (31μl) were combined to 5 μl of the other first-strand primer (SEQ ID NO:1) for a total volume of 36μl (solution B). Each of the two solutions (sol A) and (sol B), independently, was denatured at 65 degrees C for 10 min and put in two tubes (one containing denatured sol A and the other containing denatured sol B). Simultaneously, 100μl of 2X of buffer GC (TAKARA), 20μl of 2.5mM dNTPs, 40μl of saturated trehalose (approximately 80%, low metal content; Fluka Biochemika), and 4μl of Superscript II reverse transcriptase (Invitrogen) (200 u/μl) were combined to a final volume of 164μl (solution C). Further, 0.2μl of [32P]dGTP were placed in a third tube. Solution A was mixed on ice with solution C, and an aliquot (20 %) of the mixture was quickly added to the tube containing the [32P]dGTP. First-strand cDNA synthesis was performed in a thermocycler with a heated lid (MJ Research) according to the following program: step 1) 45.degree C for 2 min; step 2) gradient annealing:

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cooling to 35.degree C over 1 min; step 3) complete annealing: 35.degree C for 2 min; step 4) 50 degree C for 5 min; step 5) increase to 60 degree C at 0.1 degree C per second; step 6) 55 degree C for 2 min; step 7) 60 degree C for 2 min; step 8) return to step 6 and repeat for 10 additional cycles. Incorporation of radioactivity permitted estimation of the yield of cDNA (Carninci and Hayashizaki, Methods Enzymol. 1999;303:19-44). The cDNA obtained was treated with proteinase K, extracted with phenol/chloroform and chloroform, and ethanol-precipitated using 5M NaCl.

The same procedure carried out for solution A was performed for solution B and cDNA obtained and treated in the same way.

RNA removal.

Pellet was dissolved with 100 μl of H_2O and treated with the same volume of 150 mM NaOH / 15mM EDTA. After incubation at 45 degree C for 10 min, following solutions were added: 100 μl of 1M Tris-HCl pH7.0 (we can combine two samples on this step), 2 μl RNaseI (10U), 2 μl RNaseH (120u) (TAKARA) and incubated 37 degree C, 15min. Again sample was treated with proteinase K, extracted with phenol/chloroform and chloroform, and ethanol-precipitated using 5M NaCl. Pellet dissolved in 100 μl of water was applied to S400 column. During this step it is possible to use the same column for the samples with the same direction. Sample was precipitated with Isopropanol and washed twice with 80% of ethanol.

Part 2. Hybridization and ExoVII - Restriction Enzyme treatment.

Hybridization was carried out at Cot values of 1 to 20 in a buffer containing 40 percent formamide (from a deionized stock), 0.375M NaCl, 25 mM HEPES (pH 7.5), and 2.5 mM EDTA. Hybridization was carried out at 42 degree C. in a dry oven for 14hrs. After hybridization, the sample was precipitated by adding 2.5 volumes of absolute ethanol and incubated for 30 minutes on ice. The sample was then centrifuged for 10 min at 15,000 rpm and washed twice with 70% ethanol; the hybrids were resuspended in 90μl of water on ice.

Exonuclease VII treatment: for degradation of un-hybridized single stranded DNA was performed by addition of 10XL buffer (TAKARA) and 0.5μl of enzyme. Reaction mix was incubation at 37 degree C for 40min. Later remained hybrids were treated with proteinase K, extracted with phenol/chloroform and chloroform, and ethanol-precipitated using 5M NaCl. Sample was resolved in 85μl of TE. 5μl of sample has been used for S1 nuclease check. We added of 0.5μl 10XS1 buffer (300mM Na acetate pH 4.5, 150mM NaCl, 0.05mM $ZnSO_4$) (TAKARA) to the sample, took 2μl from the buffer-sample mixture and put on DE81 paper (Whatman) and checked the radioactivity (standard method). After that we add 2μl of enzyme S1 (30u) and incubate at 37C for 30min, took 2μl and put them on DE81 paper (Whatman), S1 sensitive rate was calculated (Carninci and Hayashizaki, Methods Enzymol. 1999;303:19-44). Restriction Enzyme Digestion was done with the addition to the reaction mix (sample 80μl, 10Xl buffer 10 μl, BSAμl) of 2μl *Hap*II and 1.5μl *Hpy*CH4IV. After incubation at 37 degree C or 2h, 2μl 5M NaCl and 1.5μl *Aci*I was applied and incubation was continued for another 2hrs. All three

random oligonucleotides (Sambrook et al. Molecular cloning Lab. Manual, CSHL press,1989) 5μl (5μg) first incubated on 94 degree C for 30sec. It was put on ice and 5μg cDNA (hybridized) were applied to the mixture on ice. Then, it was incubated at 37 degree C for 3min. room temperature and the same volume of 2XCTAB Buffer (0.4M NaCl 2mM CTAB 20mM EDTA) was added at room temperature and incubated at 45 degree C for 20min (incubation can also be carried out at 37 degrees C for 20min or at room temperature for 20min). After incubation, the sample was mixed with tRNA(Sigma) treated magnetic beads, rotated at room temperature for 30min and washed with 500μl 3M TMA Buffer (Tetramethylammonium Cloride, Sigma)(3M TMA, 20mM EDTA, 50mM Tris-HCL pH 7.5) 4 or 5 times. The radioactivity of the labeled samples was measured before and after the procedure in order to estimates the yield. 50ul of 0.25X solution containing 4M Guanidium Thiocyanate, 0.5% n-lauryl sarcosine, 25mM Sodium Citrate pH7.0 100mM beta-mercaptoethanol with 0.5% Biotin and incubated 37 degree C for 10min. Supernatant was recovered and radioactivity was measured again. Steps were repeated until 80% or more cDNA hybrid was recovered. Sample was precipitated with isopropanol and in order to remove free biotin purification for 2-3 times has been done using Sepadex G50 (Amersham Pharmacia). Here capture release step can be repeated at least once again.

Part 4. linker ligation, PCR and Cloning.

Y shaped linkers were designed with GC 3' overhangs that could ligate to 5' C/G overhands generated after the treatment of DNA hybrids with *Hpa*II, *Hpy*CH4IV and *Aci*I. 40ng/μl of ASEL9. The two strands of the Y-shaped linker were the following:
Up-5' AAAAAGCAGGCTCGAGTCGAGTCGACGAGAGAGGC (SEQ ID NO:3);

purpose only. In another embodiment of the invention the PCR reaction can be performed with 20 cycles, or in yet another embodiment with 30 to 40 cycles to obtain sufficient amount of the PCR product for the direct use of the PCR product in other application rather than the cloning only. Proteinase K digestion was conducted followed by extraction with phenol/chloroform and chloroform (Carninci and Hayashizaki, Methods Enzymol. 1999; 303:19-44), and sample was dissolved with 40μl of TE.

Cloning.

Cloning part included vector preparation (digestion and fragment purification with QIAGEN kit, QIAGEN), restriction digestion of cDNA fragmentes with *Bam*HI and *Sal*I and cloning of fragments into the vector. Vector pFLC1 (Carninci et al., September 2001, Vol.77, (1-2):79-90). was double digested with 1μl of *Sal*I and 1μl *Bam*HI using 10μl 10X*Sal*I buffer (all NEB) and 10μl of 10X BSA in total 100μl and incubated at 37 degree C for 1hr. After Proteinase K treatment, extraction with phenol/chloroform and chloroform, and ethanol-precipitation using 5M NaCl, linear fragment of the vector was resolved in 100μl of and applied on electrophoresis (0.8% NuSieve). The DNA fragment were cut out from the gel and purified by Gel Extraction kit (QIAGEN). Vector was dissolved in 100μl of water. Digestion of PCR product as also performed with 1μl of *Sal*I and 1μl *Bam*HI using 10μl 10X*Sal*I buffer (all NEB) and 10μl of 10X BSA in total 100μl and incubated at 37 degree C for 1hr. After Proteinase K treatment, extraction with phenol/chloroform and chloroform, and ethanol-precipitation using 5M NaCl, linear fragment of the vector was resolved in 100ul of and applied on electrophoresis (0.8%